THE ETIOLOGY AND DIAGNOSIS OF HYDROPHOBIA.*

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INTRODUCTION.

DURING the Spring of 1904 the "Negri bodies" were demonstrated by one of us in smears from the central nervous system of animals dead from hydrophobia. At that time, however, the technic was poor and the stains were unsatisfactory, so the use of the method in diagnosis was not begun. Many of the cases reported by Dr. Poor were studied by us in this way, the "bodies" being demonstrated in smears from three horses and from several dogs and guinea-pigs, while they were not found in normal dogs, guinea-pigs, or rabbits, or in guinea-pigs dead from tetanus or diphtheria toxin.

Last fall, in connection with the study of smears from vaccinia and variola stained by Giemsa's method, smears from hydrophobia cases were again tried and it was found that the "bodies" were brought out very clearly and characteristically by the Giemsa solution; and, as a consequence, the present work was planned.

Some of the most interesting material used by us has been obtained through the kindness of Dr. R. J. Wilson and of a number of veterinarians of New York City, to all of whom we wish to express our thanks.

We also wish to thank Dr. Poor and Dr. van Gieson for assistance in making some of the smears and sections from diagnosis animals, as well as for valuable suggestions.

Most of the sections have been prepared by Miss C. R. Gurley. The microphotographs were made in the Loomis Laboratory of Cornell University by Dr. M. Tracy, to whom we wish to express our

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HISTORICAL REVIEW.

Investigations on hydrophobia have been carried on from three principal standpoints; first, the therapeutic; second, the diagnostic; and third, the etiological. Since the establishment of the Pasteur treatment, the importance of making a quick diagnosis has become so evident that the efforts of many workers have been directed toward this end, and only occasionally has the purely etiological standpoint been considered.

Pasteur and his immediate followers relied for their diagnosis entirely upon rabbit inoculations, and this meant a 15 to 20 days' wait before the patient knew whether or not the treatment he was receiving was necessary. In 1898 this time was shortened to about nine days in our laboratory by Wilson, who found that guinea-pigs came down with the disease much more quickly than rabbits. From time to time it has been thought that certain histological findings were diagnostic; for instance, the "rabic tubercles" of Babes, and the areas of "round and oval-celled accumulation in the cerebrospinal and sympathetic ganglia" of Van Gehuchten and Nelis, were said to be specific, but further study has shown that they are not absolutely specific for rabies. In many cases the whole picture of the grosser histological changes is sufficiently characteristic to warrant the diagnosis of rabies, but often it is not so. Bailey, in his studies on the ganglion cells in normal and hydrophobic rabbits gives a good bibliography of the histological findings up to 1901.

It was not until Negri, in 1903, described certain bodies seen by him in the large nerve cells in sections of the central nervous system that anything was found which seemed absolutely specific for hydrophobia. Negri claims that these bodies are not only specific for rabies, but that they probably are animal parasites, and the cause of the disease.

He describes them as usually round or oval bodies from 1 μ to 23 μ long, and containing vacuoles in some of which are granules of varying size and number; generally there is a central larger structure surrounded by smaller ones. By Mann's method of staining, the organism generally takes a brilliant eosin-red, with the exception of the granules, some of which stain a light blue, and the others a faint red. The central structure gives the appearance of being a nucleus. The bodies are sometimes in touch with the nucleus of the host cell, sometimes far from it, often in the cell branches where they are more elliptical in shape. There are irregular, pear-shaped, and three-cornered forms, all of which special shapes Negri thinks due to the position of the organism within the host cell. He speaks of multiplying forms but does not describe any definite division forms. He says he is able to identify the bodies in the hanging-drop fresh, and in a weak acetic acid solution; but does not recommend this method for general use in diagnosis, as it is difficult to differentiate the bodies under these conditions from the nerve-tissue elements. He finds his organism generally in greatest numbers in the large nerve cells of Ammon's horn, less frequently in those of the cerebral cortex, the cerebellum, the medulla, the spinal cord, and the cerebrospinal ganglia. The organisms vary greatly in numbers in the different cases. In some cases he could find only an occasional one, while in others they were innumerable.

He says very little about the bodies in animals dying of fixed virus, merely stating

that they have been found in rabbits that have died on the seventh day after inoculation with fixed virus, but that they are very tiny, infrequent, and found with difficulty. He finds the largest forms in dogs inoculated subdurally with street virus.

Negri's work was soon corroborated by many Italian observers. Volpino, Bertarelli, D'Amato, Daddi, Di Vestea, Guarnieri, and Martinotti, published almost immediately after Negri's first publication. They were soon followed by Celli and De Blasi, Pace, and Bosc. The "bodies" have been found by these authors, and later by others in all varieties of animals which are susceptible to hydrophobia, i. e., in dogs, cats, rabbits, rats, mice, guinea-pigs, birds, cattle, horses, and human beings.

In 1904 Luzzani published a report of 179 cases, and in all but nine of those which were proven by animal inoculation to be rabies, the "Negri bodies" were found.

In our own laboratory in the same year, Poor examined 19 cases of street rabies and many cases of experimental rabies, and in all except those inoculated with fixed virus the "bodies" were found. In fixed-virus animals he found an occasional homogeneous eosinophilic granule in the cerebellar cells, about which he expressed no opinion. Similar granules were also seen by various other observers, some of whom consider them possibly tiny forms of the organism; but nothing definite has been observed about them, and as indefinite granules have also been seen in other conditions, their significance is uncertain.

In 1904 Negri's work, so far as the presence of these "bodies" in hydrophobia is concerned, was further corroborated by the following workers: Dominici, Marzocchi, Bandini, Fasoli, and Schüder. There was no dissenting voice as to their presence, and as to their diagnostic value. Many controls were made by the different observers, especially by Volpino, Marzocchi, Dominici, and Poor. They examined the central nervous system of various animals that had died from poisoning with tetanus, strychnin, pneumococcus, staphylococcus, alcohol, formalin, tubercle bacillus, diphtheria toxin; and of human beings who had died from epilepsy, syphilis, alcohol poisoning, tuberculosis, and various nervous affections. Many normal animals were also examined, all with negative results so far as the "Negri bodies" were concerned.

During this time the "bodies" were tested for their resistance to various physical and chemical agents, such as heat, cold, drying, immersion in glycerin, etc., and they were found to retain their characteristic appearance and virulence after more or less manipulation. It does not necessarily follow, however, that the "bodies," even if they are living organisms, need to retain their characteristic appearance in order to be virulent. We know, for instance, that trypanosomes may seem to disappear from blood which continues to be infective. (Laveran and Mesnil.)

Remlinger showed that the medulla of rabbits inoculated subdurally with fixed virus may be virulent on the third day, but he did not work out the exact degree of virulence—that is, the approximate number of organisms in the material inoculated. The fact that it is virulent soon after inoculation, and that no "Negri bodies" have been found at this early period, he thinks is another indication that they are not organisms. He does not consider the possibility of there being tinier forms than those so far seen, but believes that the organism in its whole life-cycle is ultra-microscopic in size.

In regard to the significance of the "bodies," up to 1905 all of these authors, with two exceptions, agree with Negri in considering them probably Protozoa and the cause of hydrophobia. The two exceptions are Remlinger and Schüder.

These latter investigators consider the fact that the virus can be filtered through

a filter, practically impervious to ordinary bacteria, a proof that the "Negri bodies," which they say are too large to pass such a filter, are not the cause of hydrophobia. Bertarelli, however, showed that the residue after filtration was also virulent, and he and others expressed the opinion that besides forms too large to pass the filter there might be forms tiny enough to do so. We know that in a medium containing a growing protozoon we may find both large and small forms, the limits in size of the smallest forms not being known in some cases; the fact therefore, that the filtered portion and unfiltered solid residue both possess virulence is an added indication that we are dealing with Protozoa. MacNeal has shown with the trypanosomes that besides the large forms, there are forms tiny enough to pass a Berkefeld.

Practically nothing has been done with regard to the exact degree of virulence possessed by filtered and unfiltered portions of the emulsions of rabies virus. Late in 1905, Di Vestea showed that the filtered virus possesses characteristics different from unfiltered, thus indicating that the forms in each may be different in character. He thinks that the undiscovered extracellular forms may be tiny enough to pass the filter.

Quite recently Volpino elaborates more fully an hypothesis advanced by him in 1904, in regard to the filterable forms. He thinks that the real organism is very tiny, that probably only the inner bodies in the so-called "Negri body"—the tiny bodies which he had shown to be definite basophilic forms—are the parasites, and that the homogeneous-appearing substance in which they are imbedded and which makes up the rest of the "Negri body" as Negri describes it, is derived from the host cell, caused by the reaction of it to the parasite. He gives a number of drawings arranged in the form of a life-cycle to illustrate this idea.

Negri's latest article, appearing in June, 1905, states that the central body shows more characteristically as a nucleus in sections from rabid cattle which he had stained in a special way by hematoxylin, and that in the same animals there appear bodies presenting characteristics of cysts. These later studies confirm all of his previous work and emphasize the fact that some of the bodies contain a central complex characteristic mass of chromatin, sometimes appearing solidly stained, sometimes as a distinct network, and sometimes encircled by smaller solidly staining masses of chromatin. Each chromatin mass is surrounded by a clear, unstained ring.

The bodies which he interprets as cysts, he describes as similar in dimensions, shapes, and general staining characteristics to the other forms, but different in minute structure. By the staining method of Mann they seem to be filled with tiny, refractive, somewhat elongated granules. Some seem to be surrounded by a membrane which is occasionally notched as if about to break. The iron-hematoxylin stain brings out the structure of these bodies very clearly. They seem to be filled with numerous black-staining "spores" less than 1 μ long and narrower, which appear as tiny filaments slightly curved with a small swelling near the center.

In 1905 still other workers corroborated Negri's work, among them Abba and Bormans, Way, Zaccaria, Maresch, Schiffmann, Galli-Valerio, and Bohne. Only one author failed to corroborate the work. Maas, in sections from a case of human rabies could find no "Negri bodies." Luzzani in this year published another collection of cases. Out of 457, 297 proved by the biological test to be hydrophobia, and in only nine of these were the "bodies" not found in sections. The bodies were not found in any other animal.

Maresch, by Bielschofsky's staining method, claims to have brought out the structure more distinctly.

Schiffmann, after studying the "Negri bodies" as they appear in street rabies and examining many controls, confirming fully the diagnostic value of the "bodies," studied the changes which they seemed to undergo in passage from animal to animal of the same species and of one species to another. He states that the greater the number of passages through a single species of animal, the smaller the "bodies," until in "fixed virus" in the rabbit no forms appear. He also says that he did not find any "bodies" in dogs inoculated with rabbit-fixed virus.

Bohne describes the shortest method so far published for examination of sections. The whole process lasts only three hours, and the author states that it is very satisfactory. The method is as follows: Small pieces of the nerve tissue are placed in 15 c.c. of pure aceton and kept at 37° C. for about 30 to 45 minutes. They are then put in 55° paraffin and left from 60 to 75 minutes, boxed, cut at 6 μ, dried at 60,° and stained with a modified Mann's method in 4 minutes. The "bodies" show a vaculated and granular structure and some of the elliptical forms seem to be dividing. On the whole they take more of a magenta stain than the "bodies" do in sections prepared in the regular way. The author considers their parasitic nature still doubtful.

During 1905 a good review of recent studies on hydrophobia came out in the *Bulletin de l'Institut Pasteur*, and in 1906 Bertarelli published a good review in the "Referate" of the *Centralblatt für Bakteriologie*.

We may sum up the results obtained from the foregoing studies as follows:

1. In nearly 100 per cent of definite cases of street rabies characteristic "bodies" are found in the large nerve cells of sections from all or from a part of the central nervous system and the connected ganglia.

2. The general characteristics of most of these "bodies" are as follows: rounded or oval forms varying in size from 1 μ to 25 μ, with a homogeneous acidophilic ground substance containing a central body surrounded by granules; these inner bodies vary in structure and staining qualities, but are principally basophilic and may be in the form of reticular masses, rings, rods, or points; they are usually situated within vacuoles.

3. The "bodies" vary also in number, being very few in some cases, and numerous in others. According to one author they become fewer the greater the number of passages through a single species of animal, and are not found in fixed virus. Others have found occasional small forms in fixed virus, but not in large enough numbers to account for the infectivity of the nerve tissue.

4. No "bodies" have been found before the appearance of symptoms, although the central nervous system is infective before this time.

5. No "bodies" have been found in the peripheral nerves or in the salivary or other glands, although these organs have been shown to possess a certain amount of infectivity.

6. The most rapid satisfactory method of demonstrating the "bodies" for diagnosis is a complicated section process which takes at least three hours.

7. The filtered virus is infective, therefore some forms of the causative agent must be extremely tiny.

8. In no other disease have bodies similar in appearance to the "Negri bodies" been found.

9. When the "bodies" are found in sections, the diagnosis of hydrophobia is certain and the biological test need not be made; when they are not found, the case may have been one of hydrophobia and the biological test must be made.

10. The significance of the "bodies" is still in doubt for the following reasons: (*a*) They have not been found in all cases of hydrophobia, notably not in fixed virus, neither have they been found in all parts of nervous tissue proved to be virulent, especially before the beginning of symptoms; (*b*) forms small enough to pass the coarser Berkefeld filters have not been seen; (*c*) the structure has not been shown definitely to be analogous to that of known living organisms; (*d*) no definite series of forms indicating growth and multiplication have been demonstrated; (*e*) the staining qualities, contrary to those of known Protozoa, are more acidophilic than basophilic.

In January of 1906 one of the writers made a preliminary communication of part of the work reported in the following pages. Emphasis was placed upon the fact that the demonstration of the "Negri bodies" by the "smear method" which was recommended by the writer in 1904 (see discussion under Poor's first article) had, by better technic, proved to be wonderfully successful. By this method the structure of the "bodies" is brought out more definitely than by the section method, and the whole process is much simplified and may be completed within half an hour after removal of the nerve tissue from the animal.

The method of examining the central nervous system, especially

the brain, by smears has been used by several pathologists, among whom may be mentioned Ewing, who obtained interesting results by this method in his studies on the pathology of ganglion cells.*

ORIGINAL WORK.

The work may be divided into two parts:

I. The value of the "Negri bodies" in diagnosis and their rapid identification.

II. A study of the "bodies" with a view to determining their nature.

In all, 141 animals, including seven varieties, have been studied with these two points in view. The following table gives a classified list of these animals:

Street rabies cases	Dogs	25
	Cats	1
	Human beings	3
Animals inoculated with street rabies	Dogs	7
	Rabbits	12
	Guinea-pigs	32
	Mice	5
Animals inoculated with fixed virus	Dog	1
	Rabbits	27
	Guinea-pigs	7
	Mice	1
Control animals	Dogs	12
	Rabbits	4
	Guinea-pigs	2
	Calf	1
	Human being	1

I.

In the first part of the work we have tried to determine: (1) Whether the "bodies" seen in the smears are similar to those seen in the sections, (2) the correspondence between the smear method, the section method, and the biological test, (3) the comparative value of each method in diagnosis, and (4) the specificity of the "bodies."

It was decided that these points might be brought out by using all three diagnostic tests in a series of street rabies animals and of a number of controls. Therefore with each animal chosen for this purpose

*Just after this paper went to press the article by Frothingham appeared. His work corroborates the results obtained by the smear method of diagnosing rabies. We have tried the impression method which he describes, as well as a number of other methods of making smears of the central nervous system and find the results obtained by them all good in some particulars, but the method we describe has so far given us uniformly better results in the diagnosis work.

the following routine was carried out: (1) the brain, medulla, and parts of the spinal cord and connected ganglia were removed; (2) small pieces from each part were fixed in Zenker's fluid; (3) smears were made from corresponding parts; and (4) animals were inoculated subdurally with an emulsion of corresponding parts, and from the animals that died either smears or sections or both were made.

The technic of the smear work is as follows:

1. Glass slides and cover-glasses are washed thoroughly with soap and water, then heated in the flame to get rid of oily substances.

2. A small bit of the gray substance of brain chosen for examination is cut out with a small sharp pair of scissors and placed about one inch from the end of the slide, so as to leave enough room for a label. The cut in the brain should be made at right angles to its surface and a thin slice taken, avoiding the white matter as much as possible.

3. A cover-slip placed over the piece of tissue is pressed upon it until it is spread out in a moderately thin layer, then the cover-slip is moved slowly and evenly over the slide to the end opposite the label. Only slight pressure should be used in making the smear, but slightly more should be exerted on the cover-glass toward the label side of the slide, thus allowing more of the nerve tissue to be carried farther down the smear and producing more well-spread nerve cells. If any thick places are left at the edge of the smear, one or two of them may be spread out toward the side of the slide with the edge of the cover-glass. If the first smear does not seem to be well spread out others should be made until a satisfactory one is obtained.

4. For diagnosis work such a smear should be made from at least three different parts of gray matter of the central nervous system: first, from the cortex in the region of the fissure of Rolando or in the region corresponding to it (in the dog the convolution around the crucial sulcus), second, from Ammon's horn, third, from the cerebellum. In many of the animals reported here smears were made from the gray matter of the cerebral cortex, around the fissures of Rolando and Sylvius, from the olfactory bulb, Ammon's horn, cerebellum, medulla in the region of the roots of the cranial nerves, spinal cord in the dorsal and lumbar regions, spinal and Gasserian ganglia, salivary glands, suprarenals pancreas, and some of the peripheral nerves. From the last four-named structures the smears were not very successful, so only a few were made.

5. The smears are dried in air,* and subjected to one or both of the two following staining methods:

(*a*) Giemsa's solution. The smears are fixed in methyl alcohol (commercial is just as good as pure) for about 5 minutes. The staining solution recommended

* This method has proved so practical in our hands that an effort is being made to extend its usefulness.

The Board of Health of New York City is preparing a circular containing a description of the foregoing technic with more explicit directions in regard to the regions from which the smears are to be made with the added information that such smears, as well as the fresh material, may be sent to the nearest laboratory familiar with the appearance of the "Negri bodies" or to the Research Laboratory of the N. Y. Health Department. If the smears have been made successfully and the "Negri bodies" are found, the sender may receive word almost immediately and no sections or inoculations of the material need be made.

last by Giemsa* (1 drop of the stain to every c.c. of distilled water made alkaline by the previous addition of one drop of a 1 per cent solution of potassium carbonate to 10 c.c. of the water) is poured over the slide and allowed to stand for one-half to three hours. The longer time brings out the structure better, and in 24 hours well-made smears are not overstained. After the stain is poured off, the smear is washed in running tap water for one to three minutes, and dried with filter paper. If the smear is thick, the "bodies" may come out a little more clearly by dipping in 50 per cent methyl alcohol before washing in water, then the washing need not be as thorough. By this method of staining, the cytoplasm of the "bodies" stains blue and the central bodies and chromatoid granules stain a blue-red or azur. Generally the larger "bodies" are a darker blue than the smaller, the smallest of all may be very light (Plate 19, Figs. 3–56). The stain varies somewhat according to the thickness of the smear. Some have a robin's-egg blue tint but this is after a longer fixation in the methyl alcohol. In this case the red blood cells may have a greenish tint. (See Part II for full description of "bodies" stained by this method.) The cytoplasm of the nerve cells stains blue also, but with a successfully made smear the cytoplasm is so spread out that the outline and structure of most of the "bodies" are seen distinctly within it. The nuclei of the nerve cells are stained red with the azur, the nucleoli a dull blue, the red blood cells a pink-yellow, more pink if the decolorization is used. The "bodies" have an appearance of depth, due to their slightly refractive qualities.

For diagnostic purposes this method of staining may be shortened as follows: Methyl alcohol, 5 minutes, equal parts of the Giemsa solution and distilled water, 10 minutes. In this way "bodies" are generally brought out well enough for diagnosis, and sometimes the structure shows distinctly. It is always well, however, to make smears enough for the longer method of staining, in case the shorter one should prove unsatisfactory.

(*b*) The eosin-methylene blue method recommended by Mallory. The smears are fixed in Zenker's solution for one-half hour; after being rinsed in tap water they are placed successively in 95 per cent alcohol+iodine one-quarter hour, 95 per cent alcohol one-half hour, absolute alcohol one-half hour, eosin solution 20 minutes, rinsed in tap water, methylene-blue solution 15 minute, differentiated in 95 per cent alcohol lasting from one to five minutes, and dried with filter paper. With this method of staining the cytoplasm of the "bodies" is a magenta, light in the small bodies darker in the larger; the central bodies and chromatoid granules are a very dark blue, the nerve cell cytoplasm, a light blue, the nucleus a darker blue, and the red blood cells a brilliant eosin pink (Plate 18, Fig. 2). With more decolorization in the alcohol the "bodies" are not such a deep magenta and the difference in color between them and the red blood cells is not so marked.

The "bodies" and the structure are often more clearly defined with this method and perhaps on the whole it is better to use it for making diagnoses;† but when there

*Azur II—Eosin . . . . . 3.0 g.
Azur II 0.8
Glycerin (Merk. chem. pure) . . . . . 250.0 c.c.
Methyl alcohol (chem. pure) 250.0

Both glycerin and alcohol are heated to 60° C. The dyes are put into the alcohol and the glycerin is added slowly, stirring. The mixture is allowed to stand at room temperature over night, and after filtration is ready for use.

The solution is prepared ready for use by Grübler, Leipzig.

† Dr. Poor recommends it strongly for diagnostic purposes.

are only tiny "bodies" present, or when the brain tissue is old and soft, the Giemsa stain seems to be the more successful; above all, when one wishes to study the nature of the centrals tuctures and granules the Giemsa stain must be used. We therefore recommend strongly the use of both methods. Even if both are used and one has to wait for the longer method, the technic is far simpler than any so far published.*

Not only do the "bodies" come out more distinctly by the smear method, but the pathological changes accompanying them are well demonstrated. For instance, the swellings of the neuro-fibrils described by Ramon y Cajal, the collections of the lymphoid cells, the increase of the endothelioid cells, the degenerated nerve cells are all clearly seen.

The technic of the section work is as follows: (1) The small pieces are left in Zenker's fluid for three to four hours; (2) washed in tap water for five minutes; (3) placed in 80 per cent alcohol+iodine (enough tincture of iodine added to give port wine color) for about 24 hours; (4) 95 per cent alcohol+iodine 24 hours; (5) 95 per cent alcohol 24 hours; (6) absolute alcohol from four to six hours; (7) cedar oil until cleared; (8) cedar oil+paraffin 52° āā, two hours; (9) paraffin 52° two hours in each of two baths; (10) boxing; (11) sections are cut at 3 to 6 μ, dried in thermostat at 36° C. for about 24 hours protected from the dust, and stained according to the eosin and methylene blue method recommended by Mallory. The most important point in the technic is the time the material is allowed to remain in Zenker. Accord ing to our experience, two hours fixation is not enough, three to four hours is very good, and with every hour after five hours the results become less satisfactory. Left in Zenker over night the tissue is granular and takes the eosin stain more or less deeply, both of which results interfere with the appearance of the tiniest "bodies," especially of the very delicate, tiny forms found by us in sections from fixed virus. Another point in favor of the short fixation in Zenker is that the precipitate formed by the mercury is not so great and is more easily got rid of, which is a very great help in the identification of the tiniest forms. Schiffmann recommends short fixation in Zenker, but he does not state the time he finds best.

It is thought, also, that washing for any great length of time in water after fixation does not help the specimens, the few that were left for a much longer time than the five minutes are not as satisfactory as the others.

In regard to the rest of the technic, it is sufficient to say that the changes to the different fluids were made with great regularity, and the final differentiation in alcohol of the stained sections was done most carefully.

In the sections made in this way we have been able to demonstrate clearly very tiny forms as well as good structure in the larger forms, a description of which will be given in Part II.

* Van Dieson working in our laboratory, suggests a staining method which differentiates the "Negri bodies" more quickly than either of the two methods described above, So far, the best proportion of the stains used have not been determined, but satisfactory results have been obtained from the following mixture: To 10 drops of distilled water three drops of a sat. alc. sol. of rose anilin violet and six drops of Löffler's solution of methylene blue are added. The smears are fixed while moist in methyl alcohol for one minute. The stain is then poured on, warmed until it steams, poured off, and the smear is rinsed in water and allowed to dry.

The cytoplasm of the "bodies" is a deep and distinctive red, their inner structures are a dark blue, the nerve cells are a light blue and the blood cells a pale salmon-red.

The staining mixture remains good for about an hour.

TABLE 1.

RESULTS OF EXAMINATION OF RABIES MATERIAL BY MEANS OF SMEARS, SECTIONS, AND ANIMAL INOCULATIONS.

No.	Species	Date of Autopsy	Clinical Diagnosis	Presence of Negri Bodies in Smears	Presence of Negri Bodies in Sections	Result of Animal Inoculation	Presence of Negri Bodies in Smears from Animals Inoculated	Presence of Negri Bodies in Sections from Animals Inoculated
		1905						
1..	Dog	11—10	Rabies	+	..	+	..	..
2..	"	11—23	"	+	+	..	..	..
3..	"	12— 2	Doubtful	+	+	..	..	..
4..	"	12— 4	Rabies	+	+	..	..	..
5..	"	12— 9	Suspicious	—	—	—	..	..
6..	"	12— 9	"	—	—	—	..	..
7..	"	12— 9	"	—	—	—	..	..
8..	"	12—15	Rabies	+	..	+	+	+
		1906						
9..	"	1— 4	"	+	+	+	+	+
10..	"	1—10	"	+	+	..	..	..
11..	"	1—18	Doubtful	—	..	—	..	..
12..	"	1—22	Rabies	+	+	+	+	+
13..	"	1—26	"	+	+	+	+	+
14..	"	1—29	"	+	+	..	..	..
15..	"	2—20	"	+	+	+	+	..
16..	"	2—23	"	+	+	+	+	+
17..	"	2—26	"	D'btful*	+†	+	+	+
18..	"	2—26	Doubtful	—	—	—	..	..
19..	"	2—27	Rabies	+	..	+	..	..
20..	"	3— 2	"	+	+	+	+	+
21..	"	3— 3	"	+	+	+	+	+
22..	"	3— 6	Distemper or Rabies	+	+	..	..	..
23..	"	3—12	Rabies	+	+	+	+	+
24..	"	3—13	"	+	+	+	+	+
25..	"	3—26	"	+	+	..	..	..
		1905						
26..	Cat	12— 5	"	+	..	+	..	..
27..	Human	11—10	"	+	+	+	+	..
28..	Child	11—16	"	+	+	+	+	..
		1906						
29..	"	1—16	"	+	+	+	+	+
30..	Human	1— 9	Alcoholic neuritis	—	—	—	..	..
31..	Dog	1— 4	Inoculated with human rabies. No symptoms	—	..	..	..	..
32..	"	1—16	Inoculated with human rabies. No symptoms	—	—	..	..	..
33..	"	1—30	Inoculated with human rabies. Typical symptoms	+	+	..	..	..
34..	"	1—31	Inoculated with human rabies. Typical symptoms	+	+	+	..	..
35..	"	2— 6	Inoculated with human rabies. Typical symptoms	+	+	+	..	..
36..	"	2—15	Inoculated with human rabies. Typical symptoms	+	+	+	+	+
37..	"	3— 6	Inoculated with street rabies. Typical symptoms	+	..	+	+	+
		1905						
38..	Calf	11— 5	Normal	—	..	..	..	..
39..	Dog	11—13	"	—	..	..	..	..
40..	"	11—14	"	—	..	..	..	..
41..	"	11—14	"	—	..	..	..	..
42..	"	12— 1	"	—	..	..	..	..
43..	"	12— 1	"	—	..	..	..	..
44..	"		"	—	—	—	..	..

* Brain in bad condition. Two days old.

† A few tiny "bodies" found.

In Table 1 we have given the results of the animals studied with a view of determining the four points mentioned at the beginning of this section. In some of them the full examination as planned was carried out, in others, besides the smears, only sections or animal inoculations were made. The controls are not as many as we might have made had not so much control work been done previously by us and by so many others.

The results are as follows:

1. No control animal shows appearances similar to the "Negri bodies," either in smears or in sections. The various suspicious cases, especially the case of the dog with filaria, we consider among the best controls, because here we are dealing with animals dead after symptoms similar to those of hydrophobia.

2. In all of the cases proved by the biological test to be hydrophobia, "Negri bodies" are found in either smears or sections or in both.

3. In the animals which had been inoculated from these animals, "Negri bodies" are found in either smears or sections or in both.

4. The general characteristics of the "bodies" seen in the smears are similar to those of the "bodies" seen in sections.

5. The three tests correspond as to diagnostic results.

6. The smear method is much better than the section method in demonstrating the "bodies" for diagnostic purposes.

7. When the "bodies" are present in the smears the diagnosis of hydrophobia is certain, even if the biological test is negative. When they are not found the diagnosis is uncertain.

8. In a very few cases of street rabies, only extremely tiny forms are found. These may be easier to find in sections than in smears.

9. In doubtful or negative cases both the section method and animal inoculations should be tried.

II.

In studying the nature of these bodies many points have only been touched upon and others are still being investigated, but we believe that enough new knowledge has been gained to warrant this publication. The plan of this part of the work is as follows:

1. The comparison of the general characteristics of the "bodies" { in smears. / in sections. / in hanging-drop.

   a) Size { in different species of animals. / in different animals of same species.
   b) Shape { in different parts of same animal.
   c) Number { in different stages of the disease.
   d) Site { in different numbers of passages.
   e) Structure { after different modes of inoculation.

2. Detailed characteristics of structure.
   a) Cytoplasm.
   b) Central bodies.
   c) Chromatoid granules.
   d) Different shapes.
   e) Division forms { transverse. / longitudinal. / budding.
   f) Conjugation forms.
   g) Stages at which different forms appear.

3. Relation between the time the central nervous tissue becomes infected and the time the bodies appear.
4. Spread of the bodies to different parts of the host.
5. Significance of the bodies and comparison with known organisms.
6. Summary.

1. *General characteristics of bodies in smears compared with those in sections: Size.*—The majority of the forms seem larger in smears than they do in sections from the same case. The largest forms measured are about 18 μ and the smallest structured forms about 0.5 μ. We can easily see that a form appearing as 0.5 μ in a smear might scarcely be visible in a section, and that such tiny forms, considering their extreme plasticity (see under structure), might easily pass the coarser Berkefeld filters. We have found that the size varies more with the course of the disease (which includes the question of accustoming the virus to the host, e. g. fixed virus), than it does merely with different species of animals. This means that the bodies may vary greatly in different animals of the same species in different parts of the same animal and at different stages of the disease. We may say in general that no very large forms are found in the early stages

of the disease or in any stage in certain varieties of especially susceptible animals to which the virus has become accustomed (fixed virus). While in later stages of the disease in animals inoculated with virus from another species, or in varieties of animals that are not fully susceptible to the disease, both large and small forms are found.

We have not yet had the opportunity of examining smears from rabid cattle, so we are not able to corroborate the statement of Negri that the largest forms are found in this variety of animal; but if it holds, it would seem that the reasons for the fact might be that cattle are among the less susceptible animals, and that they are generally inoculated with a virus from a different species of animal. Of course, other things being equal, we should expect a certain amount of variation in size and structure of an organism growing in different species of animals, just as we get variations in the same variety of bacteria and of other low forms of life grown in different culture media.

Shape.—The shape of the bodies appears more varied in smears than in sections, due partly to the fact that there is a certain amount of distortion. The distortion, however, is very slight, because within narrow limits of disturbance (i. e., too much or too unequal pressure in making the smears) the bodies are broken up and their identity lost. The principle types of shapes seen in smears are given in the accompanying plates. Plate 19, Figs. 3 to 56 inclusive, and the photographs may be studied in this connection. The same types of shapes are seen in all varieties of animals studied.

Number.—Generally more bodies are seen in smears than in sections from similar parts of the same case. Since we have learned to identify many tiny bodies, we have found that there are more in all cases, including fixed-virus cases, than have hitherto been reported. In any case we feel that we are able to demonstrate enough forms, or, at least, to account for enough forms, to correspond to the degree of infectivity of the part.

Site.—As is shown in Plate 18, Fig. 2 the topography of the bodies may be well preserved in smears. Their situation in the cytoplasm of the body and branches of the larger nerve cells is well shown. In parts of the smear which are more broken up the bodies may appear as if lying free, and it is these bodies, if the pressure has not been too great, that show the structure best. Such bodies have for the most part been

chosen for the photographs (especially 1, 2, 4, and 5). There are often many tiny "bodies" in degenerating nerve cells, but these show better in sections than in smears. The tiny forms which we have seen in the nuclei of the host cells also appear more distinct in sections than in smears.*

Structure.—The principal point in favor of the smear method of examination is that the structure of the bodies comes out so clearly and so characteristically that it is easy to draw a close analogy between it and that of known Protozoa. In the first place, as has been shown by Negri and most of the other investigators, the following fact holds true: Whatever the variety or species of animal infected, the bodies preserve their same general characteristic structure, i. e., a hyaline cytoplasm with an entire margin, and with one or more inner bodies having a more or less complicated and regular structure. This fact alone, that by such an entirely different method of examination the bodies show the same characteristic structure in so many different varieties of animals, is a very strong point in favor of their not being degeneration forms.

In general we may say the same things in regard to the relation between structure of the "bodies" and the variety, etc., of the animal, that we did when discussing size, because their structure varies to a certain extent with their size. The tiny forms, rounded, with a more or less centrally-situated chromatin-staining granule, slightly larger forms with three to several such granules (often four), elongated forms with a central chromatin line, and tiny forms in two or in groups of three or more (Plate 19, Figs. 3–8) are the only types found in fixed virus (with an occasional slightly larger form containing a larger central body and a few tiny granules). The tiny forms found in fixed virus seem to be far more delicate than apparently equally tiny forms seem in other lesions, that is, they take the stain more delicately, the central structure is not so distinct, and the whole body is more easily destroyed by pressure in the former than in the latter case. Hence, it is only in the best made smears that these fixed-virus forms are seen, and then only after the eye has been accustomed to their very delicate coloring and outline.

The forms found in fixed virus animals are the only ones which

* With van Gieson's new staining method these tiny forms are well differentiated in smears.

are better preserved or at least which are more distinctly seen in sections than in smears. This is due probably to their extreme delicacy. The fact that we have found very many forms in all cases (15) of developed fixed-virus infection studied makes it probable that they are present in every case and that they come out better with the technic described in Part I than with the technic followed by other investigators. In regard to their specificity, we would say that we have made few controls for the following reason. As slight alterations in technic seem to interfere with their demonstration, and as, therefore, their non-appearance might not mean that they are not present, large numbers of animals would have to be examined before one could be sure that forms simulating them might not be present in certain cases. The facts, however, that in our four controls and in the first two days after inoculation of a series of ten experimental rabbits (see below for details of this experiment), they are not found, and that when they do appear they possess certain characteristics, in structure, site, and number corresponding to the course of the disease, makes it pretty evident that we are dealing with the specific organism. These bodies have the following characteristics: They are tiny rounded forms, sometimes wavy in outline, as if possessing slight amœboid motion, sometimes elongated, extending along the rim of the host-cell nucleus, or along one of the nerve fibrils, as if moving there; they take a delicate light magenta stain very similar to that taken by the small serum globules in the blood vessels, and it would be difficult, if not impossible, to distinguish some of them from these serum globules, if they were in the blood vessels. Many of the organisms, however, show a small chromatin granule, situated more or less eccentrically, sometimes on the very rim of the body. In the larger forms the granule is large, in the smaller it cannot always be seen (Plate 18, Fig. 1); some of the larger forms show from two to several granules and occasionally there is a body with the definite central body and the the small granules about it. In these fixed-virus sections we have found certain tiny bodies in some of the nerve-cell nuclei, especially in the smaller of those cells which show decided degenerative changes of the cytoplasm. These intranuclear forms seem to stand out quite distinctly from the rounded, acid-staining degenerative masses. The latter are not so refractive as the former. The intranuclear forms have not yet been studied suffi-

ciently to allow a decided opinion in regard to their place in the life-history of the organism. They are quite frequent in the olfactory bulbs of guinea-pigs after inoculation with rabbit-fixed virus.

The fact that none of the larger forms of the "bodies" are found in animals dying after fixed-virus inoculations is an added indication that the bodies are not products of degeneration of the host cells.

That the development of only these tiny forms with their simple structure in fixed-virus animals is due to the fact that the special strain inoculated is accustomed to the one variety of host is shown by the result obtained by inoculating the strain into another variety of animal. We have inoculated one dog and several guinea-pigs subdurally, and three mice subcutaneously with fixed virus from the rabbit, and in each case (in only one case in mice, as only one of the three died) besides the tiny forms there have been numerous large forms with the characteristic, definite, more or less complicated structure (corresponding to Plate 19, Figs. 17–34). This is contrary to the results obtained by Schiffmann upon inoculating rabbit-fixed virus into dogs. In his cases he could find no bodies whatever. On the other hand, we have had delayed fixed-virus action in one rabbit (inoculated with 2 c.c. of a thin emulsion into the ear vein, with death on the 11th day after typical symptoms of paralytic rabies), and in this animal we found only the tiny, delicate forms found in the other fixed-virus rabbits.

In regard to variations in structure at different stages of the disease, most of our study has been made upon animals inoculated with fixed virus, and the forms and structure in these cases seem to be about the same in the early stages as in later ones. It would seem that under these favorable conditions for the organism it grows and divides so rapidly from the beginning, and infects so many of the host cells that the animal is overwhelmed before the parasite has a chance to develop the larger forms. The results are different in the animals inoculated with street virus.

We inoculated one series of seven rabbits with street virus from a dog, killed the first animal on the seventh day after, and the others respectively on the 9th, 11th, 12th, 14th, 16th, and 17th days. The results as to number and structure of the bodies are briefly as follows:

Seventh-day rabbit.—In the bodies of the large nerve cell of Ammon's horn and cerebral cortex an occasional tiny form and an occa-

sional one of the intermediate grades were seen. (Forms corresponding to Plate 19, Figs. 3–16.)

No definite extracellular forms were seen, but neither sections nor smears have yet been studied minutely. This is the earliest day reported for forms found after inoculations with street virus. Negri reports finding them on the 10th day in a dog. In our series of animals those that were allowed to remain alive did not begin to have visible symptoms until the 13th and 14th days.

Ninth day rabbit.—Many very definitely structured forms were seen in the large nerve cells of practically all parts of the cerebral nervous system, smears and sections showing equally well. The forms corresponding to Plate 19, Figs. 3–12 were in the majority, those corresponding to Figs. 13–16 in moderate numbers, and those corresponding to Figs. 17–32 occasionally.

Eleventh-day rabbit.—Practically no difference between it and 9th day one.

In the *12th, 14th, 16th,* and *17th day rabbits* the larger forms appeared in gradually larger numbers and many more division forms were seen.

So far most of the study in this series has been made on the earlier stages.

There are no marked differences in the "bodies" found in different parts of the central nervous system of one animal dead 25 days after inoculation into the sciatic nerve. The general histological lesions are more intense in the cord and there is a larger number of the larger "bodies" there than usual, but the "bodies" in the brain are about the same in number and structure as in animals dying from subdural inoculations.

Appearance of "bodies" in hanging drop.—So far, we have done only enough work with the hanging drop to make us realize that it is an extremely difficult method of study and needs most careful control at each step. There is no doubt that certain forms of the organism can be recognized; but the nerve tissue elements change so quickly, assuming flagellated and delicately granular form which simulate those of known organisms that the control must be at one's side before one realizes that the object studied is not a living organism.

Detailed characteristics of structure.—In smears as well as in sections, the *cytoplasm* appears quite homogeneous, there is no evidence of a reticulum, or of a granular structure outside of the definite chromatoid granules. The smears, however, have brought out one important point in regard to the cytoplasm more clearly than the sections, and that is that it is more basophilic than acidophilic in staining qualities. With the Giemsa stain, as we have seen in Part I, it takes the methylene-blue stain more than the eosin-red, and even with the simple eosin methylene-blue stain the protoplasm appears as a deep magenta unless much decolorized.

One of the points, then, which has been brought up against the protozoan theory falls to the ground. The cytoplasm takes the stain as does that of many well-known protozoa—the malarial organism, for instance.

In studying the *central bodies* of these organisms, as they appear in the smears, one of the first things noticeable is that they are not surrounded by a clear space—that there is no sign of a vacuolar appearance in the whole body. This is a very different appearance from that given in the sections, and it shows that the vacuoles described in the sections are artefacts due to the technic. We notice next that in the great majority of the organisms the central body stands out clearly, as decidedly different in structure, and slightly so in staining qualities, from the chromatoid granules which surround it. The general type of the structure of the central body is that of well-known protozoan nuclei; for example, Prowazek gives a description of the nucleus in certain stages of the *Plasmodiophora brassicae*, which might be used here to describe the most typical appearance of these central bodies.

The chromatin is arranged in a more or less granular ring around the periphery of the central body or nucleus leaving an achromatic or more acid-staining center in which is situated, generally eccentrically, a varying-sized karyosome (Plate 19, Fig. 37). There are a number of variations from this principal type, according to stage of development. Often the whole nucleus answers to the description of the compound karyosome given by Calkins in his description of the protozoan nucleus. In the tiny "bodies" the chromatin can only be seen as a dot, in those a little larger it may be a large solidly staining gran-

ule, or a ring or rod, the latter often hour-glass shaped. In forms large enough for the characteristic structure to be developed and to be clearly seen, the central body may show evidence of fragmentation (Plate 19, Figs. 18, 38, 51, etc.). Just such evidence of fragmentation is shown in many protozoan nuclei preparatory to division. It is interesting that forms showing this phase, and, moreover, very similar in general appearance to some of the forms seen here, have been depicted by Doflein in the early stages of the life-cycle of *Glugea lophii*, a myxosporidium, parasitic in the ganglion cells of a fish (*Lophius piscatorius*).* The staining of the nucleus will be considered with that of the chromatoid granules.

The chromatoid granules are most frequently arranged in a more or less complete circle about the nucleus. They are somewhat irregular in outline and size, being occasionally ring-shaped, sometimes elongated, often in twos, due probably to active changes of growth and division. They take generally a more mixed chromatin stain than the chromatin of the nucleus. This fact is brought out in the Giemsa-stained smears. Here the nuclear chromatin takes generally a definite azure tint, while the chromatoid granules are more of a blue, though sometimes they may appear more red. That the red in the central body and granules is not an eosin-red, is shown first by its peculiar magenta tint, and second by the fact that when partly decolorized by methyl alcohol, the red color disappears from these structures leaving them a dark blue, while the cytoplasm is a pale blue-pink and the red blood cells are a definite eosin-pink. If a dilute methyl alcohol is used, an interesting series of differentiations in color may be obtained. Such a more or less regular arrangement of chromatoid granules in the cytoplasm of Protozoa is of frequent occurrence (Calkins, Minchin). It is a marked feature, according to the observations of one of us, in certain stages of the *Plasmodiophora brassicae*. The further changes in the central bodies and granules will be considered under division forms.

Different shapes.—We agree with Negri in considering many of the different shapes due to the position of the organism in the host cell. There is no doubt that the substance of these bodies is extremely delicate and plastic, easily adapting itself to the position in which it

* In Doflein's later classification (1901) he names this species *Nosema lophii* and places in the suborder *Microsporidia* under the order *Cnidosporidai*.

is found and easily destroyed by artificial means. Many of the elongated forms are forms growing and dividing in this way because of position between the fibrils. The triangular forms (Plate 19, Figs. 26, 50, and Plate 21, Fig. 7) are probably forms that have grown in the angle made by the giving off of a nerve cell branch. They have been placed by us, in Plate 19, underneath the much elongated forms as possible division forms of the latter; but they probably are not. The principal cause of most of the different shapes, however, is the rapid growth and division of the organism.

Division forms.—The whole picture is one of rapid growth and multiplication, and this corresponds with the clinical history. The elongated forms containing from two to five or even six nuclei are the result of rapid nuclear division without corresponding cell division. This condition is found quite frequently in Protozoa (*Thelohania Mulleri*, Minchin, p. 292). The elongation in this way is probably due, as we have said, to the position of these bodies between the nerve fibrils, and to their great plasticity.

Under the most favorable conditions (fixed virus), growth and division occur most rapidly and simply, the tiny forms dividing and redividing apparently indefinitely. Whether there is simple conjugation, or fusion of unequally divided forms during this condition, it is difficult to say. It would probably take much study to settle this question. Small mulberry masses are found during this stage, but whether they are the result of the breaking up of a larger form or of the rapid division of a tiny form it is impossible for us to say as yet. We have also seen appearances which suggest plasmodial phases. There seems to be distinct evidence of an intranuclear invasion also in fixed-virus infection.

In cases where there has been an inoculation of comparatively small quantities of the virus, i. e., a small number of forms of the parasite capable of immediate infection, or in cases where there has been an infection of less susceptible animals (dogs, cattle, human beings, etc.), or with a less accustomed virus (fixed virus of rabbits into guinea-pigs or mice), we get a slower growth with its larger structures and different division forms. The chromatin accumulation in the form of a definite nucleus, apparently undergoes fragmentation very easily, and so we have forms containing two to several central bodies,

some rounded (Plate 19, Figs. 12, 13, 14, 19, etc.), some elongated (Fig. 15), some of unequal division, similar to budding (Fig. 29). Then we find forms with bodies apparently differentiated within one membrane (Figs. 20, 31, 53), and bodies with practically all stages of hour-glass constriction, indicating transverse division (Fig. 32). Many pairs, unequal in size, apparently fusing or dividing have been seen (Figs. 33, 45), and finally, we have large bodies with the chromatin scattered throughout the whole organism in the form of tiny, unevenly rounded or elongated masses, one or two larger, indicating the remains of the nucleus, and in these forms we get all stages of apparent budding (Figs. 40, 41, 42, 54, 55). The buds vary somewhat in size, some being very tiny. The formation of buds accounts for the appearance in the same cell of both large and small forms. It also helps to account for the rapid spread of the organisms. These tiny budded forms similar to "swarm spores" are probably motile and pass quickly to other host cells.

We have also found a number of more or less indefinite masses, taking the stain a little more deeply than the other bodies, and apparently made up of large numbers of tiny bodies, but so far they have been too indefinite for us to be sure that we have cystlike structures We have not studied the sections minutely enough yet to find out how such structures appear there, or whether they are similar to the "cysts" described by Negri.

Conjugation forms.—At first sight "the buds" were thought by us to be possibly conjugating individuals, but when on further study they were found to be principally, if not entirely, in forms which showed marked fragmentation of the chromatin, they were interpreted as budding forms. Such unequal forms as are represented in Plate 19, Figs. 33 and 45 may be conjugating forms, but so far we have not been able to decide as to their significance.

The relation between the time the central nervous tissue becomes infective, and the time the bodies appear.—Our principal work on this point has been done with fixed virus. After finding that tiny, characteristic forms were found in two rabbits dying on the eighth and ninth days after subdural inoculation with fixed virus, we inoculated 10 rabbits subdurally with fixed virus (629th passage), killed one every day by chloroform, and examined the central nervous system in the

following way: One-half of the brain and medulla, including the olfactory bulb, was cut into slices, and with slices from the dorsal and lumbar spinal cord, including one or two spinal ganglia, was placed in Zenker, and subjected to the technic for sections mentioned in Part I. From the other half of the brain, and corresponding parts of the cord, two sets of smears were made, and each stained respectively by the two methods mentioned in Part I. Unfortunately, with this series of animals, we did not test the virulence of the nerve tissues, so we do not know at exactly what period it became distinctly virulent. However, in an earlier series of eight rabbits inoculated in the same way, and from which only smears were made, Dr. Poor tested the virulence roughly, as follows: One animal was killed each day, with the exception of the eighth, which died on the ninth day. From the lumbar cord, and from Ammon's horn, pieces of about the same size, so far as we could judge from eye measurement, were cut. Two dilutions were made from each piece, a stronger one, by the addition of 3 c.c. of normal salt solution, making an emulsion; and a weaker one, by making a 1:1,000 dilution of the stronger. Two guinea-pigs were inoculated with the weak dilution $\frac{1}{2}$ c.c. each; two with the strong dilution, $\frac{1}{2}$ c.c. each.

Of the animals inoculated with the weak dilutions of the *cord*, none died; of those inoculated with weak dilutions of the *brain*, none died from the first or second day rabbits, one died from the third day, and one from the fourth day animal, none from the fifth day, one from the sixth day, two from the seventh day, and none from the ninth day animal. Of the animals inoculated with the strong dilutions of the *cord*, none died from first, second, third, and fourth day rabbits; one from the fifth day, one from the sixth day, and none from the seventh day animal. Eighth and ninth day animals were not inoculated. Of the animals inoculated with the strong dilutions of the *brain*, none died from first and second day rabbits, two died from the third, fourth, fifth, sixth, and seventh day rabbits, eighth and ninth day animals not inoculated.

In this experiment, then, the weak dilution of the *cord* was not infective in the doses used; the strong dilution was not infective until the fifth day, and then not regularly so; while both dilutions of the *brain* became infective on the third day, the weaker one less so, and

continued so to the end. These results corroborate the work of Remlinger, who found the medulla virulent on the third or fourth day after subdural inoculations of fixed virus.

In neither of these sets of experiments has the approximate number of organisms present been shown, and until we know this we cannot say that in any measured amount of infective material there may be more than an occasional tiny form, which it might be very difficult, perhaps impossible, to find in sections or smears of such material.

In the examination of the 10 rabbits mentioned first in this connection, although we have so far studied only a comparatively few sections, we have found the bodies appearing as follows: On the first, and second days none; on the third day an occasional one in the large lymphoid cells of the perivascular lymph spaces at the base of Ammon's horn; on the fourth day, a few tiny undoubted ones in the large nerve cells of the olfactory bulb, of the lower curve of Ammon's horn, and of the motor area of the cerebral cortex; on the fifth, a moderate number in the same areas and in scattered cells throughout the whole brain; on the sixth, many in the same areas, and in the medulla; on the seventh (two animals), on the eighth, and on the ninth, very many, as in the other fixed-virus animals studied (Plate 18, Fig. 1).

From this series of experiments it seems that the bodies may be found soon enough and in practically large enough numbers to account for the beginning infectivity of the nerve tissue, and that with only a little more careful experimenting this may be brought out clearly.

Four control rabbits were studied in this connection; two normal rabbits, one which had died from pneumococcus infection, and one from yeast infection.

Spread of the bodies to different parts of the host.—This point is now being studied by us. It is taken up under two heads; first, the spread of the organisms from the point of inoculation, and second, its spread from the site of infection.

In whatever way the virus enters the body, so far as we know, there is no development of the organism, or none, to any appreciable extent, until it reaches the central nervous system, and not until after a certain amount of development there does it infect the peripheral organs. Before the disease was well studied it was thought that the salivary glands were the chief site of the infection. But it has been shown

that these glands are not always infective, and when they are, not until comparatively late in the disease and that when the virus is inoculated into them, the animal seldom comes down with the disease and probably never if the centripetal nerves are cut (Bertarelli). This means that the parasite does not grow in the salivary glands, that it is only carried there incidentally by its spread from the central nervous system along the nerve branches. That the organisms escape into the blood and are carried in this way in small numbers is shown by the fact that the blood in large quantities has been found infective (Marie). Principally by the nerve channels, secondarily by the blood and lymph channels, the organisms are carried in small numbers to all parts of the body. With other investigators, we have found the suprarenal capsules infective (in one out of two street-rabies dogs). One of the three guinea-pigs inoculated died after typical symptoms of rabies, and the central nervous system showed many good-sized bodies and was infective for other animals. If it is true that the organisms pass in such comparatively small numbers to the various peripheral organs, and especially if only the smaller forms pass, then our chances of identifying them in the salivary and other glands are very slight. Smears from these parts are unsatisfactory, and we have not yet been able to study the sections.

In regard to the spread of the organisms from the point of inoculation, the parasites are probably carried to the central nervous system along channels similar to those by which they are carried away, and unless enough of them can quickly reach the nerve cells, they are probably destroyed by the macrophages. We have found, as we have said, what appear to be tiny bodies in the large lymph cells on the third day after inoculation with fixed virus. In one fixed-virus rabbit, found dead on the morning of the seventh day after inoculation, an animal which had been used before, and whose resistance was probably lessened, the central nervous system was loaded with large lymphoid cells many of which were apparently filled with tiny organisms. This question is still being studied.

Significance of the "bodies" and comparison with known organisms. —Although it may be questioned whether enough forms have been found to account for every stage in a life-cycle, it is certain that the great majority of the bodies stand out so clearly as organisms with such

definite, constant, characteristic structure and staining reactions and show so many forms similar to division forms of known Protozoa, that the picture is difficult to explain in any other way than as that of a developing organism belonging to the group Protozoa. It seems unnecessary further to consider the possibility of their being changed red blood cells or any other form of degeneration of the host tissue; and this alone is evidence in favor of their being organisms.

From time to time cases have occurred in which the "bodies" are seen in such numbers and in such stages of development that we are as sure of their being organisms as we are that the bodies photographed by Wright from Delhi boil, are organisms. As we study the picture further and find at almost every step analogies in the life-cycle of known Protozoa, the evidence is so overwhelming that there seems no reason to doubt that they are living organisms; the small single forms with their tiny chromatin central bodies rounded, elongated, or in twos and more, as in *Nosema lophii* and other Microsporidia (Doflein); the groups of small forms in twos and more (multiplicative reproduction of Doflein); the appearances of the central body in the larger forms similar to that of many protozoan nuclei at corresponding stages of development (Calkins, Prowazek); the many evidences of division of these larger forms such as fragmentation of the nucleus (Calkins), two nuclei, all stages in hour-glass constriction of the body; and finally, the distribution of the nuclear material throughout the whole organism with evidences of its fragmentation and of budding, a phenomenon which has been described as occurring in all classes of Protozoa (Calkins, Minchin)—all these and more make a collection of evidence which amounts to proof.

The parasite seems to possess more points of resemblance to organisms belonging to the sub-order Microsporidia, than to those of any other order.

SUMMARY AND CONCLUSIONS.

1. The smear method of examining the Negri bodies is superior to any other method so far published for the following reasons: (*a*) It is simpler, shorter, and less expensive; (*b*) The Negri bodies appear much more distinct and characteristic. For this reason and the preceding one, its value in diagnostic work is great; (*c*) The

minute structure of the Negri bodies can be demonstrated more clearly; (*d*) Characteristic staining reactions are brought out.

2. The Negri bodies as shown by the smears as well as by the sections are specific to hydrophobia.

3. Numerous "bodies" are found in fixed virus.

4. "Bodies" are found before the beginning of visible symptoms—i. e., on the fourth day in fixed virus, on the seventh day in street virus, and evidence is given that they may be found early enough to account for the appearance of infectivity in the host tissues.

5. Forms similar in structure and staining qualities to the others, but just within the limits of visible structure at (1,500 diam. magnification) have been seen. Such tiny forms, considering the evidence they give of plasticity, might be able to pass the coarser Berkefeld filters.

6. The Negri bodies are organisms belonging to the class Protozoa. The reasons for this conclusion are: (*a*) They have a definite, characteristic morphology; (*b*) This morphology is constantly cyclic, i. e., certain forms always predominate in certain stages of the disease, and a definite series of forms indicating growth and multiplication can be demonstrated; (*c*) The structure and staining qualities as shown especially by the smear method of examination resemble that of certain known Protozoa, notably of those belonging to the sub-order Microsporidia.

7. The proof that the "Negri bodies" are living organisms is sufficient proof that they are the cause of hydrophobia; a single variety of living organisms found in such large numbers in every case of a disease, and only in that disease, appearing at the time the host tissue becomes infective in regions that are infective, and increasing in these infective areas with the course of the disease can be no other, according to our present views, than the cause of that disease.

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DESCRIPTION OF PLATES 18 AND 19.

All figures are drawn from the object and are equally magnified (about 1,200 diameters). Objective, Zeiss apochromatic 16° millimeter. Apert. 130; Occular, Zeiss compensating 6; drawn-tube 16.

Abbreviations:

N.—Nucleus of nerve cell
br.—Branch of nerve cell
D.—Degenerated nerve cell
bv.—Blood vessel
rb.—Red blood cell
B.—"Negri body"
C.—Central body of "Negri body"
K.—Karyosome
ch.—Chromatoid granules

FIG. 1.—Section (about 5 microns) through large pyramidal cells of Ammon's horn of rabbit which was chloroformed while dying on *eighth* day after subdural inoculation of fixed virus (426th passage). Fixed in Zenker, and stained with eosin and methylene blue, according to the method described in text. In the cytoplasm of nearly every large nerve cell is situated one (sometimes two or three) tiny "Negri body," which is often near the nucleus and is sometimes elongated, extending along the nuclear membrane. These tiny bodies take a faint magenta-pink stain in contradistinction to the red blood cells, which stain a brilliant eosin-red, and most of them show within their substance a minute dark blue granule situated more or less eccentrically; sometimes at the extreme periphery of the body. In some of the smaller bodies these granules cannot be seen, probably on account of their extreme minuteness.

FIG. 2.—Smear from Ammon's horn of dog chloroformed while dying on 20th day after subdural inoculation with human rabies. Fixed in Zenker's fluid and stained with eosin and methylene blue after the method described in text.

Both bodies and branches of some of the large, blue-staining nerve cells show quite distinctly, while others are more or less destroyed. Definitely within the bodies and branches of some of these nerve cells and lying in the neighborhood of others, are seen the magenta-staining "Negri bodies" of various sizes and shapes with their central "bodies" and "chromatoid granules" staining a dark blue. The small blood vessel lying across the center shows the red blood cells staining an eosin red.

FIGS. 3 to 56, inclusive.—Some of the forms of "Negri bodies" seen in smears stained with Giemsa's solution, and arranged according to possible modes of growth and multiplication. The cytoplasm of each body stains a homogeneous pinkish blue, darker in the larger forms, very light in the tiny ones, the central body and the chromatoid granules stain a bluish red, the latter generally more blue than the former.

FIG. 3.—Tiniest stuctured form seen.

FIG. 4.—Elongated central body.

FIG. 5.—Two central bodies.

FIGS. 6, 7, and 8.—Apparent division forms of 5.

FIG. 9.—Form containing a larger, solidly staining, central body.

FIG. 10.—Form containing a ring-shaped central body.

FIGS. 11, 12, 13, 14, and 15.—Apparent division forms of 10.

FIG. 16.—Small body containing central body and chromatoid bodies.

FIGS. 17 to 33, inclusive.—Larger "Negri body" and possible division forms from it.

FIGS. 34, 35, and 36.—Bodies containing larger central body and smaller chromatoid granules.

FIGS. 37 to 55, inclusive.—Still larger forms with their possible divisions. The frequent ring-shaped arrangement of the chromatin in the central body and the karyosome-like structure within it are more apparent.

FIGS. 38 to 42, inclusive.—Apparent fragmentation of the chromatin and the formation of buds.

FIGS. 54 and 55.—Show similar budding from different forms. In Fig. 55 the elongation of the chromatin granules is marked.

FIG. 56.—Form apparently made up of many tiny indefinite bodies.

PLATE 18.

Fig. 1.

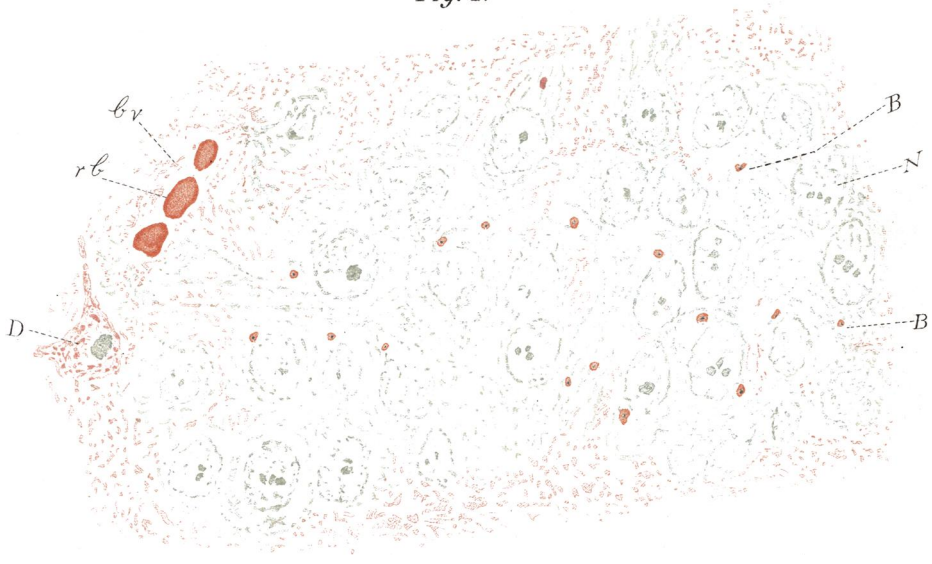

Fig. 2.

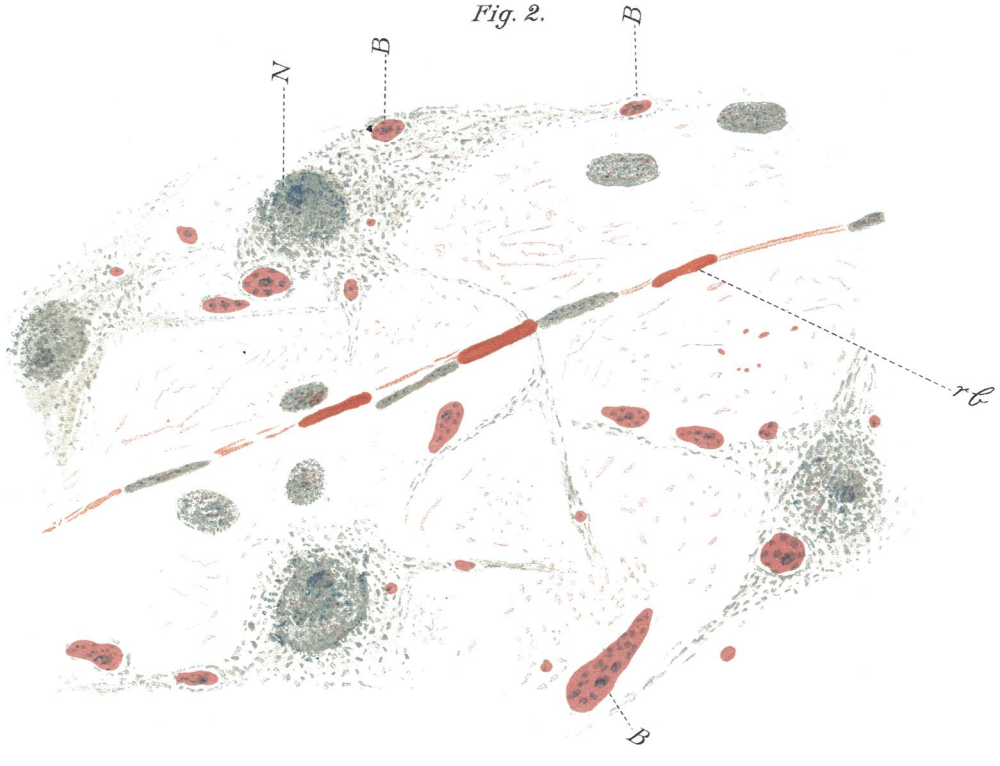

PLATE 19.


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PLATE 20.

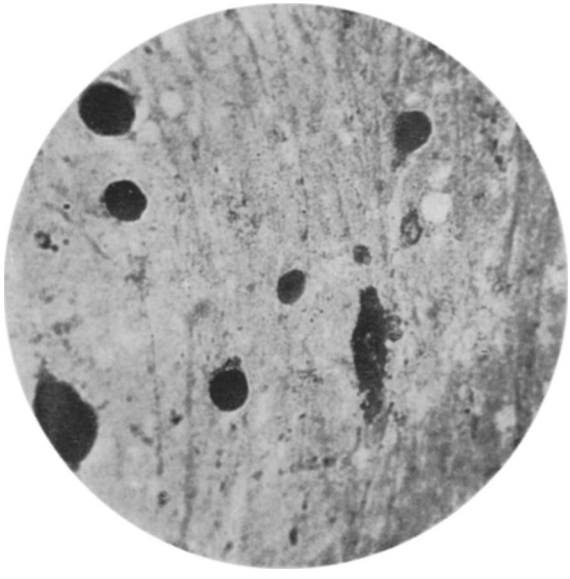

FIG. 1.

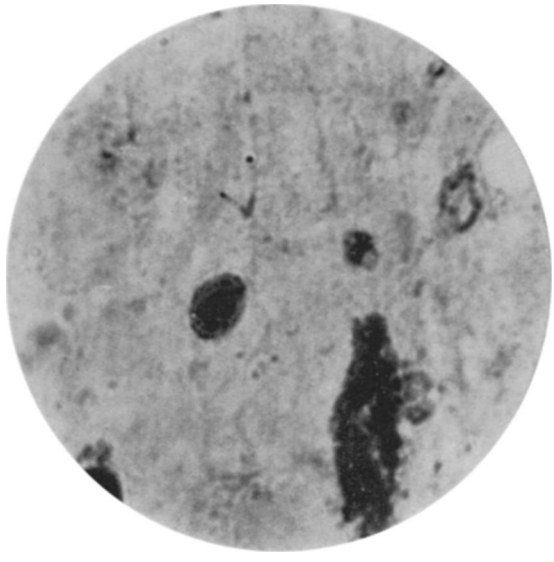

FIG. 2.

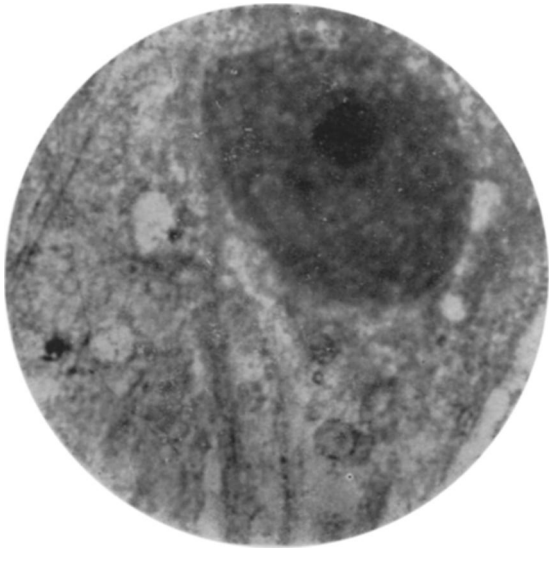

FIG. 3.

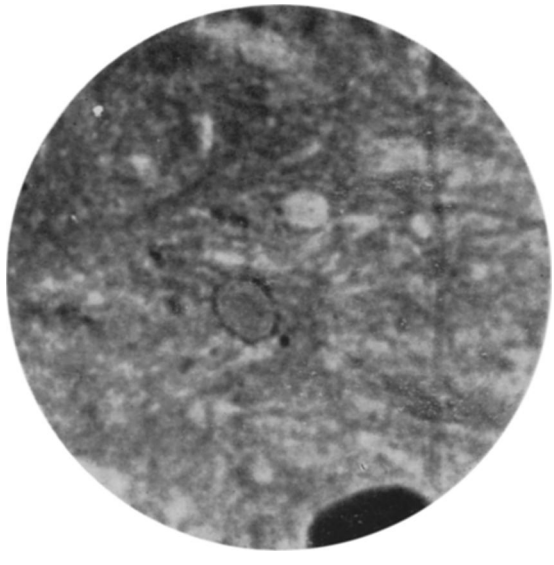

FIG. 4.

PLATE 21.

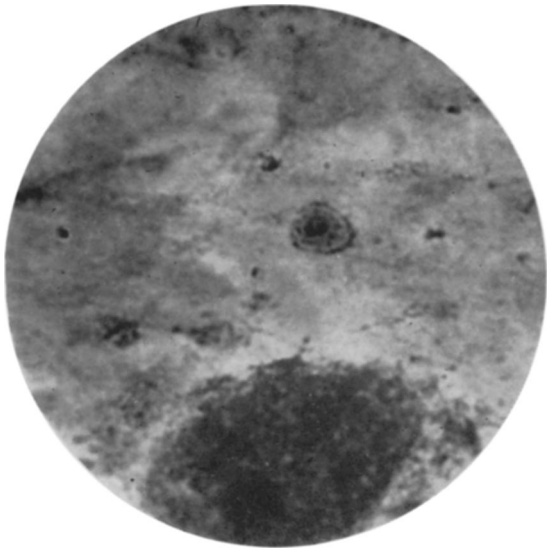

FIG. 5.

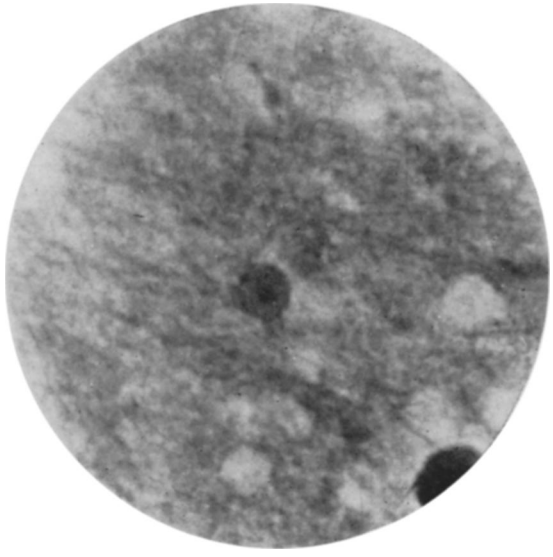

FIG. 6.

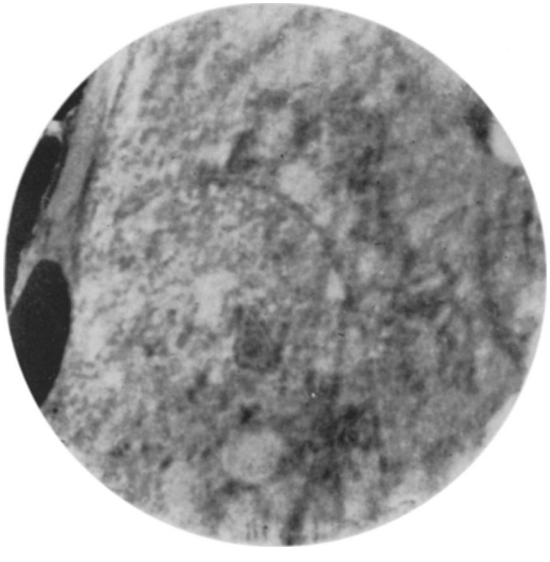

FIG. 7.

FIG. 8.

DESCRIPTION OF PLATES 20 AND 21.

All the photographs were taken from smears stained with Giemsa's solution.

FIG. 1.—From Ammon's horn, street rabies, oval "Negri body" showing central body and chromatoid granules. ×1,200.

FIG. 2.—The same body. ×2,000.

FIG. 3.—Two small structured "Negri bodies" in cytoplasm near nucleus of nerve cell. One slightly out of focus. From Ammon's horn, street rabies. ×2,000.

FIG. 4.—An oval "Negri body" pressed out from branch of cell shown in Fig. 3. The "body" contains large oval central body about which is ring of small elongated chromatoid granules. ×2,000.

FIG. 5.—A rounded "Negri body," showing well the complete circle of chromatoid granules about the central body. From street rabies. ×2,000.

FIG. 6.—Another rounded form from the same case.

FIG. 7.—A triangular form showing central body and irregular chromatoid granules, from same case.

FIG. 8.—Budding form, from Ammon's horn of dog which died 20 days after subdural inoculation of human rabies.